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STRUCTURE-ACTIVITY RELATIONS FOR CAFFEINE: A COMPARATIVE STUDY OF THE INOTROPIC EFFECTS OF THE METHYLXANTHINES, IMIDAZOLES AND RELATED COMPOUNDS ON THE FROG'S HEART

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SUMMARY

1. The ability of several groups of compounds, related to caffeine, to induce contractures in isolated frog auricular trabeculae has been tested.

2. Of the methylxanthines, theophylline, theobromine and paraxanthine are of similar potency to caffeine. This applies to contractures produced in either high-potassium or in sodium-free solution, and to the twitch responses in normal Ringer.

3. Xanthines in which the 9-position nitrogen is combined and is, therefore, without an ethylene bond do not affect contraction.

4. The hypothesis is put forward that a double-bonded nitrogen, in an imidazole ring, is required for activity of the methylxanthine. This hypothesis is supported by the ability of imidazole and several close derivatives (e.g. histamine), as well as imidazolines, to evoke contractures. As predicted by the hypothesis, imidazolidines and imidazolidones, in which all the nitrogen atoms have single bonds, fail to initiate tension development.

5. The activity of histamine and histidine is only demonstrable at high pH ($\simeq$ 9·0).

6. Raising the pH in sodium-free solution induces a transient contracture.

7. Several arguments suggest that cyclic AMP is probably not an intermediate in the response to the methylxanthines. The activity of cyclic AMP (and adenosine) in eliciting contractures is predicted by the hypothesis because they contain an imidazole moiety as part of their molecular structure.

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INTRODUCTION

Caffeine is a trimethylated xanthine and shares a variety of its pharmacological properties with the other naturally occurring methylxanthines, theophylline and theobromine. Pickering (1893) reported the effects of these chemicals on the heart of chick embryos, whilst Heathcote (1920) stated that these alkaloids increase the frequency of the rabbit heart beat and induce a brief period of increased diastolic tone. Recently, Blinks, Olson, Jewell & Braveny (1972) found that all three compounds potentiate several parameters of contraction in cat papillary muscle by increasing the duration of the action potential. Only these methylxanthines have been tested for their effect on contraction of cardiac muscle, apart from the experiments in which paraxanthine has been used as an antithyroxine (Carter, Mann, Harley-Mason & Jenkins, 1943). In skeletal muscle, caffeine is extensively used to induce contractures and its action has been studied in some detail (Lüttgau & Oetliker, 1968; Sandow, 1965, 1970). Even in this tissue, however, other methylxanthines have only rarely been used (Goutier, 1949*a*, *b*; Goffart & Goutier, 1950).

In skeletal muscle the action of caffeine has been traced to its ability to release calcium from the sarcoplasmic reticulum (Weber & Herz, 1968; Weber, 1968), a property also shown by theophylline (Johnson & Inesi, 1969).

The interpretation of these findings in relation to heart muscle is complicated by the behaviour of the 3′,5′ cyclic adenosine monophosphate system. Since methylxanthines, and particularly theophylline, inhibit the phosphodiesterase that hydrolyses 3′,5′ cyclic AMP (Butcher & Sutherland, 1962), it has been proposed that the effects of these alkaloids on cardiac contraction are mediated by changes in the cellular levels of the cyclic nucleotide (e.g. Kukovetz & Pöch, 1972; Skelton, Karch, Houghen, Marcus & Epstein, 1971; Marcus, Skelton, Prindle & Epstein, 1971).

The previous paper describes that large contractures can be induced in frog heart by quite moderate concentrations of caffeine (Chapman & Miller, 1974). The amplitude of these caffeine contractures, initiated after the spontaneous relaxation of contractures induced by perfusion with potassium-rich or sodium-depleted solutions, has a relatively simple relationship to the concentration of the drug. This observation suggests a technique for testing the relative potency of compounds similar in structure to caffeine. In the present work, therefore, the contractile responses produced by other methylxanthines, imidazoles, imidazolines, imidazolidines and imidazolidones are compared to those induced by caffeine. This comparison was motivated by two considerations: first, in the hope that it would show that the alkaloid's activity, in interfering with some

specific chemical reaction within the muscle, could be identified with a component or property of the molecule and secondly, if this activity could be identified, and some inactive relatives of caffeine found, then these inactive compounds could be tested on vesicles of sarcoplasmic reticulum from other muscles. By this means it may be possible to demonstrate whether or not the sarcoplasmic reticulum of frog heart is functionally similar to that of other muscles.

A preliminary report of part of this work has already appeared (Miller & Chapman, 1972).

METHODS

The method of isolating, mounting, perfusing and recording the tension developed by the frog atrial trabeculae was as described in the preceding paper (Chapman & Miller, 1974).

The experimental perfusion solutions are shown in Table 1. A wide variety of chemicals, obtained as listed below, were added to the perfusing media, as concentrated stock solutions or as solid. When over 10 mM of one of these substances was

TABLE 1. Composition of the physiological solutions

	NaCl (mM)	KCl (mM)	Na_2HPO_4 (mM)	NaH_2PO_4 (mM)	TrisHCl (mM)	LiCl (mM)	Glucose (mM)	pH
Phosphate Ringer	115·0	3·0	0·8	0·2	—	—	5·0	7·3
Tris Ringer	115·0	3·0	—	—	2·0	—	5·0	7·3
Sodium-free Tris Ringer	—	3·0	—	—	126·8	—	5·0	7·3
High-K Ringer	115·0	100·0	0·8	0·2	—	—	5·0	7·3
High pH sodium-free Tris Ringer	—	3·0	—	—	209·0	—	5·0	9·0
Sodium-free lithium Ringer	—	3·0	—	—	2·0	115·0	5·0	7·3
High-pH sodium-free lithium Ringer	As the sodium-free lithium Ringer with additional lithium hydroxide to adjust the pH						—	9·0

The calcium was added as 1 M volumetric standard (B.D.H.) solution.

used in an experiment a compensating adjustment of the Ringer was made to all experimental solutions so as to maintain the tonicity. Caffeine, theobromine, theophylline, pilocarpine HCl, DL-histidine, xanthine, histamine, adrenaline hydrogen tartrate, atropine, acetylcholine HCl, procaine HCl, and pyridine were obtained from British Drug Houses (B.D.H.); imidazole, 1-methyl imidazole, 4(5)-amino, 5(4)-imidazole carboxamide HCl, 2-methyl imidazole, hypoxanthine, tetracaine HCl and EGTA (ethyleneglycol-bis-(β-amino-ethyl ether) *N,N* tetra-acetic acid) from Sigma; 1,3,9-trimethylxanthine, 1,9-dimethylxanthine, 1,7-dimethylxanthine, 7,9-dimethylxanthine betaine, 9-methylxanthine, and 3-methylxanthine from Fluka, A.G.; tolazoline HCl, antazoline HCl, phentalomine and naphazoline NO_3 from Ciba; 2-imidazolidone, and 1-ethyl, 2-methyl benzimidazole from Ralph Emanuel; 2-methyl imidazoline, dibenamine, 5,6-dimethyl benzimidazole from Koch Light; adenosine and N^6 monobutryl 3′,5′ cyclic AMP from Boehringer Corp. The following chemicals were gifts and we gratefully acknowledge them: G. D. Searle for 1-methyl, 3-isobutyl-xanthine; Hoffmann-La Roche for 4(3,4-dimethyloxybenzyl)-2-imidazolidone and

4(3-butoxy-4-methoxybenzyl)-2-imidazolidone; May & Baker for promethazine HCl, and Mepyramine; Parke Davis & Co. for diphenhydramine HCl. We are also greatly indebted to Dr David Smith, Department of Chemistry, University of Leicester for synthesizing 1-hydroxymethyl-2-imidazolidone; 1,3-dihydroxymethyl-2-imidazolidone and 1,3-dimethyl imidazolidine.

These compounds include some strong acids and bases, the pH was checked and if necessary corrected after the addition of any trial compound to the perfusing solutions. This procedure was made essential by the discovery that changes (particularly increases) in the pH of the perfusing fluids could initiate contractures.

Determination of the surface tension of the experimental solutions

The method used was based on the classical glass slide technique, where the force required to just free a glass cover-slip from the surface of a solution is measured with a torsion balance. A reservoir containing the solution is raised until the cover-slip, suspended from the arm of the torsion balance, is captured by the liquid surface. The force exerted by the torsion balance is then increased while keeping the beam of the balance horizontal by adjusting the height of the reservoir. The 'weight' is recorded when the cover-slip breaks free from the meniscus. The cover-slip is then weighed in air. The surface tension of the solution in the reservoir can then be calculated from the following formula:

$$\text{surface tension} = \frac{(F-W)g}{2L},$$

where F is the force in grams weight required to lift the cover-slip free from the solution, W is the weight of the cover-slip in the air, g is the acceleration due to gravity and L is the length of one side of the cover-slip, in this case 1·8 cm.

RESULTS

Comparison of the effects of various alkylated xanthines on contraction

The basic chemical structure of the xanthines is shown in Fig. 1*a*, caffeine is 1,3,7-trimethylxanthine. Other methylxanthines potentiate the twitch responses in normal Ringer and induce contractures when applied after the spontaneous relaxation of contractures initiated by potassium-rich or sodium-depleted Ringer in a similar way to caffeine (Fig. 2).

In order to compare the relative potency of the various xanthines (as well as other compounds) a potency factor has been devised. This factor expresses the size of the contracture or the peak of the potentiation of the twitch response as a percentage of the response resulting from the application of the same concentration of caffeine under the same experimental conditions and in the same preparation. This factor is useful in illustrating the relative potency of the various chemicals but the actual value can be misleading because the dose–response curves are unlikely to be linear (Chapman & Miller, 1974). Details for the xanthine compounds are given in Table 2 for twitches and contractures.

Theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) all markedly potentiate

the twitch response and induce large contractions. 1-methyl, 3-isobutylxanthine evokes contractures that develop more slowly than those produced by the other alkaloids although the final amplitude is similar. The order of potency of the methylxanthines is, caffeine = paraxanthine > theobromine > theophylline. In contrast, 1,3,9-trimethylxanthine, 1,9-dimethylxanthine, 7,9-dimethylxanthine betaine, 9-methylxanthine, xanthine and hypoxanthine were without effect on the contractile responses,

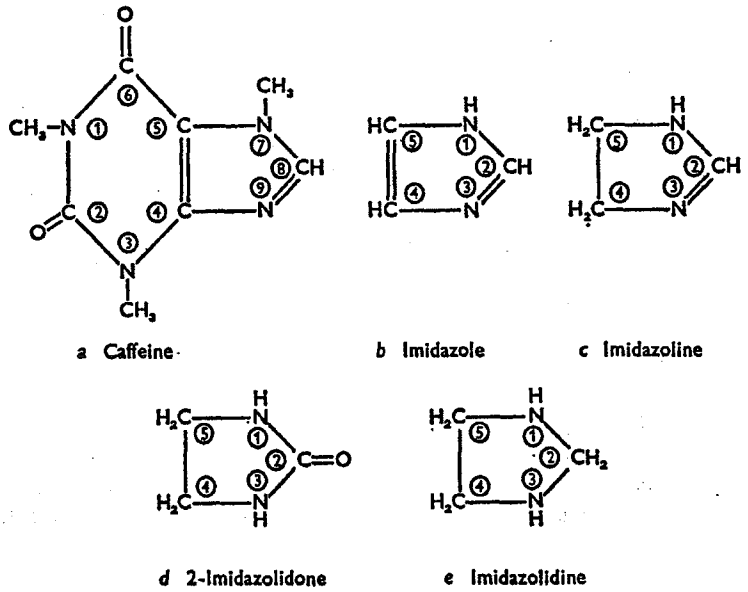

Fig. 1. The chemical structure and numbering of the atoms of a member of each group of the chemical compounds tested in the present work.

even at concentrations as high as 20 mM (as allowed by the solubility) (Fig. 2).

The consistent finding of these experiments is that all the methylxanthines that have their 9-position nitrogen combined (i.e. methylated, except xanthine and hypoxanthine which have hydrogen combined at this position at physiological pH) fail to effect or affect the contraction of the heart.

The high sensitivity of the atrial trabecula to caffeine, following the spontaneous relaxation of low sodium contractures (Chapman & Miller, 1974), has been exploited in the present work to test the efficacy of the other compounds investigated. The use of the sodium-free condition has two advantages; one during the exposure to caffeine, the extracellular

calcium concentration has no effect on the size of the contracture, and two, under these conditions, large changes in membrane potential produce only a weak tension response (Chapman, 1973*a*; Chapman & Miller, 1974).

During perfusion with sodium-free Ringer the contractures evoked by each of the active methylxanthines are of almost identical amplitude and time course, over the whole effective range. This is demonstrated by comparing the responses to mixtures of various proportions of two active equally potent methylxanthines, while keeping the total methylxanthine

TABLE 2. Twitches and contractures for compounds of xanthine

Xanthine	pK_a	Caffeine factor		
		High-K	Zero-Na	Twitches
Caffeine; 1,3,7-trimethylxanthine	0·8	100	100	100
Theobromine; 3,7-dimethylxanthine	1·08 10·0	97·5 ± 2·5	98·0 ± 6·0	96·0 ± 6·0
Theophylline; 1,3-dimethylxanthine	1·0 8·6	87·0 ± 4·6	89·7 ± 9·1	93·0 ± 3·0
1,7-dimethylxanthine	8·7	103·0 ± 2·0	97·5 ± 2·5	—
1-methyl, 3-iso-butylxanthine	—	—	100·0 ± 5·0	—
1,3,9-trimethyl-xanthine	1·0	No response	No response	—
1,9-dimethyl-xanthine	3·2	No response	No response	—
7,9-dimethylxanthine betaine	12·1	No response	No response	—
Xanthine	0·8 7·7 12·0	No response	No response	—
Hypoxanthine	1·9 8·96 12·2	—	No response	—

(pK_a's were obtained from Perrin, 1965.) The mean caffeine factors, derived as given in the text, are shown ± S.D. 'No response' corresponds to an average tension change of less than 2% of the caffeine control contractures, on application of the test substance, to a number of different preparations.

concentration constant. Performing this type of experiment over a steep part of the dose–response curve ensures that small changes in potency would be revealed. The amplitude of the contracture produced is found to be almost constant; the small fluctuations observed are paralleled by the differences in the size of the preceding sodium-free contracture (Fig. 3*a*). The presence of an inactive (9-methylated) xanthine does not alter the

dose–response curve for the contractures evoked by a contracture-inducing alkaloid (Fig. 3*b*). Contracture tension is, therefore, dependent on the total active methylxanthine concentration.

As caffeine and its relatives have mild surfactant properties, and a high rate of penetration into cells (Bianchi, 1962), the contractures observed in the presence of these compounds may be due to some detergent-like action, on either the surface or intracellular membranes. This possibility cannot be ignored, because Clark (1913) showed that soaps and other substances which reduce the surface tension of aqueous

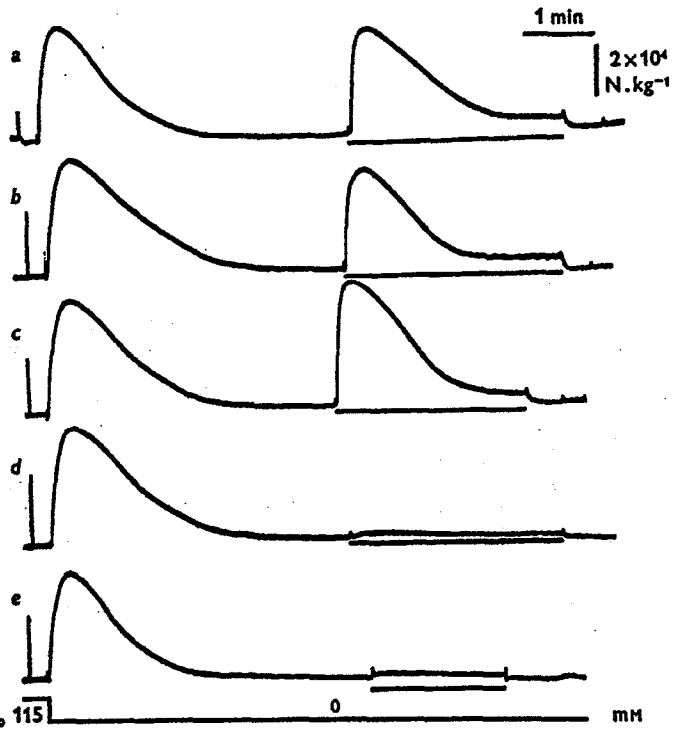

Fig. 2. After the spontaneous relaxation of a contracture induced by sodium-free perfusion, the addition of 2 mM caffeine (*a*), 2 mM theophylline (*b*) and 2 mM paraxanthine (*c*) all evoke contractures, while 2 mM 1,3,9-trimethylxanthine (*d*) and 2 mM 1,9-dimethylxanthine (*e*) are without effect. The horizontal bars under each tension trace indicate the period of exposure to each methylxanthine; 2 mM calcium; 20·0° C; Tris Ringer.

solutions have profound effects on the twitch response of frog ventricle. Measurements of the fall in surface tension of Ringer solution containing one of several of the methylxanthines, both active and inactive in producing tension, are listed in Table 3. Both active and inactive, at a concentration of 2 mM, reduce the surface tension by about 10%. This result is inconsistent with an exclusively surfactant mechanism for the action of caffeine on the frog's heart.

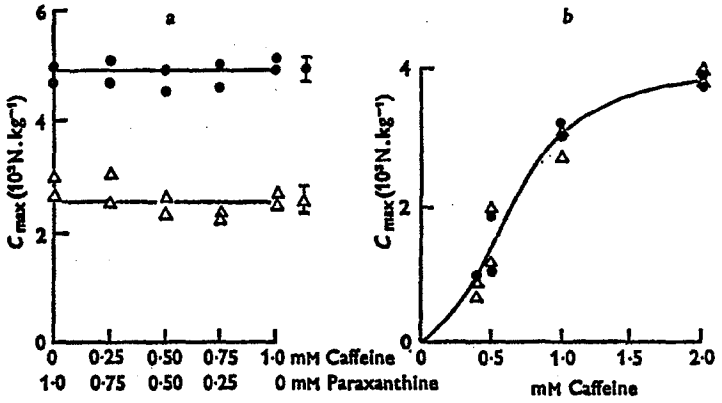

Fig. 3. The effects of mixing two active methylxanthines (*a*), and of mixing an active and an inactive methylxanthine (*b*), in sodium-free solutions, on the strength of the contracture induced by their application. *a*, compares the amplitude of the preceding sodium-free contracture (●—●) with the response induced by mixtures of caffeine and paraxanthine in which the total methylxanthine concentration was kept constant at 1 mM (△—△). Note that there is only a small variation in the amplitude of both types of response; the symbol on the left of each curve shows the mean ± S.D. of all the results for each type of contracture. 1 mM calcium; 19·0° C; Tris Ringer. *b*, from another experiment. The dose–response curve for the caffeine contracture evoked after the spontaneous relaxation of a sodium-free contracture (●—●), is not altered by the presence of 1 mM 1,3,9-trimethylxanthine (△—△). Line drawn by eye. 2 mM calcium; 19° C; Tris Ringer.

TABLE 3. Effect of methylxanthines on surface tension

Methylxanthines	Surface tension (20° C) (dynes cm^{-1} ± S.D.)
1,3,9-trimethylxanthine	67·5 ± 0·5
1,3,7-trimethylxanthine (caffeine)	64·25 ± 0·7
1,7-dimethylxanthine (paraxanthine)	68·25 ± 1·0
7,9-dimethylxanthine betaine	65·1 ± 0·5
1,3-dimethylxanthine (theophylline)	63·9 ± 0·5
Distilled water	73·6 ± 0·9

All substances were dissolved in high-potassium Ringer at 2 mM. The value for distilled water may be compared to that of 72·75 (20° C) quoted for water (Weast, 1971). Five to seven determinations were made for each mean value. The values of the surface tension of the high-potassium Ringer and of distilled water are not significantly different.

The contracture evoking ability of imidazole-like compounds

If the 9-position nitrogen in the imidazole ring of the xanthine molecule does indeed confer contracture evoking ability to caffeine, then it would be

anticipated that imidazole-like compounds should show similar properties (for the structures see Fig. 1*b*). A range of imidazoles have been tested for their ability to evoke contractures after the spontaneous relaxation of the contracture induced by perfusion with zero-sodium solutions. All the imidazoles tested were less potent than caffeine, as shown in Table 4, with 1-ethyl, 2-methyl benzimidazole proving the most effective. Histamine and histidine were generally unable to evoke contractures, and as these chemicals have an uncombined nitrogen in the imidazole ring, their inactivity would seem to conflict with the conclusions drawn from the experiments with methylxanthines. All the imidazoles tested have alkaline pK_a's (see Table 4) and therefore at physiological pH practically all the histamine or histidine is ionized. As charged molecules generally penetrate cells slowly this could account for the failure of these substances to elicit contractures, as well as for the reduced potency of the other imidazoles. On the other hand, the charged nature of these substances could interfere with their reactivity, for the ability of imidazoles to release calcium from isolated sarcoplasmic reticulum increases with the pH, whereas the potency of the methylxanthines does not (R. A. Chapman & N. G. Rutherford, unpublished). The effect of the high pK_a values can be partially overcome by raising the pH of the bathing solution but Chiarandini, Reuben, Brandt & Grundfest (1970) find that changing the pH alters the responsiveness of crayfish muscle to caffeine, while Lorkovic (1967) found no effect in frog skeletal muscle. If, following the spontaneous relaxation of the contracture, the pH of the sodium-free solutions is raised to 9·0, a contracture is induced (Fig. 4). Similar pH-induced contractures have been described for crayfish skeletal muscle fibres (Reuben, Brandt & Grundfest, 1967). The origin of these contractures is unlikely to be due to variation of the membrane potential, because the amplitude of a contracture induced by raising the potassium concentration after the zero-sodium contracture is small, particularly when the calcium concentration is low (Chapman, 1973*a*). The pH-induced contracture also develops when lithium chloride is substituted for sodium chloride, thereby excluding any effects due to unionized Tris in the Tris-HCl Ringer. Caldwell (1958) showed that intracellular buffers are able to withstand an extracellular pH change for several minutes; however, the relatively large surface area to volume ratio of frog heart cells (auricle muscle cells diameter, *ca.* 2 μm; Miller, 1973) could mean that the delay is reduced in these heart cells. A change of the internal pH as a result of the external pH step could release calcium ions from the sarcoplasmic reticulum (Nakumura & Schwartz, 1972). On the other hand, the increase of pH could alter the permeability of the surface membrane, because the pH contracture fails to develop in the virtual absence of extracellular calcium ions (R. A. Chapman & D. Ellis, unpublished).

When tension has again stabilized following the pH-induced contracture application of histamine, histidine or 4(5)-amino, 5(4)-imidazole carboxamide evoke large contractures and the size of the contracture elicited by imidazole is nearly doubled (Fig. 4 and Table 3). The response to caffeine is generally unaffected by increasing the pH of the bathing fluid, indicating that a pH-induced change in the permeability of the cell membrane cannot be exclusively responsible for the increased activity of the imidazoles.

The contracture evoking abilities of the imidazolines

The imidazole moiety of the compounds whose activities are described above, has a resonant structure due to the number of double bonded carbon atoms present. The imidazolines are simpler, having only one ethylene bond, although they are still capable of tautomerism (see Fig. 1). The imidazolines include several compounds which are known to act on the circulatory system and the heart (Scholz, 1945). Five of these compounds, naphazoline, antazoline, tolazoline, pilocarpine and phentalamine, can initiate contractures in sodium-free solutions. Their potencies, which vary widely, are summarized in Table 4. A difference in action has also been noted for their effects on the circulation (Scholz, 1945).

A simpler imidazoline, 2-methylimidazoline, is weakly active at pH 7·3 but its potency is increased at pH 9·0

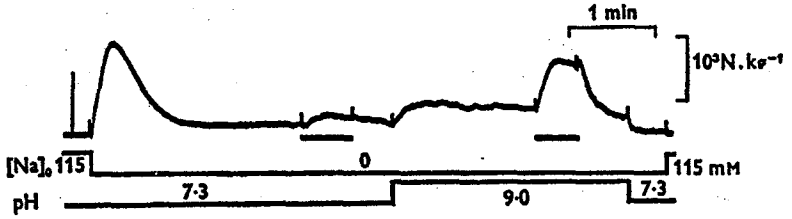

Fig. 4. After the spontaneous relaxation of the contracture induced by withdrawal of sodium from the bathing medium (as indicated by the middle trace), the application of 20 mM histamine (at the first horizontal bar) induces only a small contracture. Increasing the pH from 7·3 to 9·0 (as shown by the lower trace) initiates an increase in tension. A second application of 20 mM histamine (at the second horizontal bar), at this elevated pH, now induces a strong contracture. 2 mM calcium; 20·0° C; Tris Ringer.

The activity of imidazolidones and imidazolidines

The imidazolidones and the imidazolidines have saturated five-membered rings with both nitrogen atoms combined, i.e. they lack ethylene bonds to the adjacent carbon atoms (Fig. 1). The three simple imidazolidones and one imidazolidine tested failed to initiate significant contractures when applied to frog atrial trabeculae after the spontaneous relaxation of the sodium-free contracture. Two benzyl-2-imidazolidones did, however, induce

weak contractures in two of five preparations. Raising the pH to 9·0 has no effect on the potency of these compounds (Table 4).

The activity of other compounds containing a C = N group

Imines, non-cyclic compounds containing a nitrogen atom ethylene-bonded to an adjacent carbon atom, are generally unstable. The only

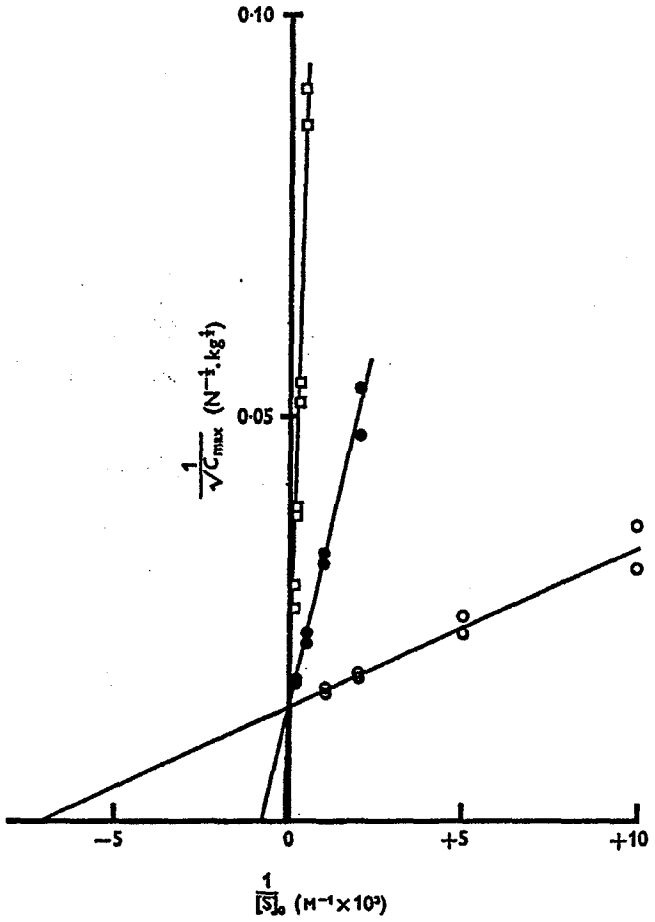

Fig. 5. $1/\sqrt{C_{max}}$ for the contractures evoked by caffeine (○—○), naphazoline (●—●) and 1-methyl imidazole (□—□), in sodium-free fluid, are plotted against the reciprocal of the concentration of the substance in each case. The common intercept with the ordinate indicates a common mode of action for the three substances in the initiation of contraction. The intercept with the abscissa yields an estimate of the affinity constant for each compound. All the lines were obtained by regression analysis ($r > 0·98$ and $P < 0·01$). 1 mM calcium; 17·0° C; Phosphate Ringer.

TABLE 4. Comparison of contractures initiated in sodium-free solution by different substances

Compound	pK_a	Caffeine factor for zero-sodium contractures (when the responses to caffeine = 100) ± S.D. pH 7·3	pH 9·0
Imidazoles			
Imidazole	6·92	17·0 ± 8·7	22·8 ± 3·8
1-methyl imidazole	7·3	6·7 ± 1·7	—
5,6-dimethyl benzimidazole	6·09 12·52	13·5 ± 1·5	—
1-ethyl, 2-methyl benzimidazole	6·55	50·0 ± 0·25	—
Histamine	6·05 10·10	No response	20·6 ± 4·4
Histidine	1·82 6·0 9·17	No response	12·3 ± 1·6
4(5)-amino, 5(4)-imidazole carboxamide	10·4 10·8	4·5 ± 1·5	24·9 ± 1·9
Imidazolines			
Pilocarpine	1·63 7·05	36·3 ± 7·4	—
Naphazoline; 2-(1-naphthyl, methyl) imidazoline	—	70·8 ± 12·2	—
Tolazoline; 2-benzyl-2-imidazoline	—	9·8 ± 2·5	—
Antazoline; 2-(*N*-benzyl-anilino methyl)-2-imidazoline	2·37 10·13	29·4 ± 2·6	—
Phentolamine; 2-(*N*(m-hydroxyl)-*p*-toiluidino-methyl) imidazoline	—	10·3 ± 4·1	—
2-methyl imidazoline	11·1	No response	16·0 ± 5·0
Imidazolidones			
1-hydroxymethyl-2-imidazolidone	—	No response	No response
2-imidazolidone	—	No response	No response
1,3-dihydroxymethyl-2-imidazolidone	—	No response	No response
4(3,4-dimethoxybenzyl)-2-imidazolidone	—	No response	No response
4(3-butoxy-4-methoxy-benzyl)-2-imidazolidone	—	No response	No response
Imidazolidines			
1,3-dimethyl imidazolidine	—	No response	No response

TABLE 4 (*cont.*)

Compound	pK_a	Caffeine factor for zero-sodium contractures (when the responses to caffeine = 100) ± S.D.	
		pH 7·3	pH 9·0
Others			
Acetamidine	12·0	No response	No response
Adenosine	—	12·7 ± 7·4	—
N^6 monobutyryl 3,5-cyclic AMP	—	9·3 ± 5·6	—
Pyridine	—	No response	No response

The pK_a values are those given in Perrin (1965). The 'no response' category was as defined in Table 2.

compound of this type tested on the frog heart is acetamidine which is totally inactive at both pH 7·3 and 9·0.

Pyridine, with an uncombined nitrogen atom in a six-membered ring, is also unable to produce contractures.

A direct comparison of members of each active class of compounds

A second-order relationship exists between the caffeine concentration and the contracture tension it initiates when applied in sodium-free solutions (Chapman & Miller, 1974). By plotting the reciprocal of the square root of the contracture tension against the reciprocal of the bathing caffeine concentration, the half-saturation constant can be derived. A similar second-order relationship is obtained in experiments using imidazoles and imidazolines; the double reciprocal plot for a member of each group of the active compounds (i.e. a methylxanthine, an imidazole and an imidazoline) yields a common intercept with the $1/\sqrt{}$(tension) axis (Fig. 5). This common intercept, which corresponds to the V_{max} of a Michaelis–Menten reaction, suggests that each compound is involved in the same chemical reaction that finally results in the development of a contracture. As the half-saturation values depend upon $[Ca]_o$, all experiments are carried out at 1 mM-$[Ca]_o$ (see Chapman & Miller, 1974). The half-saturation values for the various compounds are very different, the mean values and S.D. being, for caffeine 0·26 ± 0·08 mM, for naphazoline 3·15 ± 1·70 mM, and for 1-methyl imidazole, 24·4 ± 4·8 mM.

Caffeine can induce contractures following the spontaneous relaxation of a sodium-free contracture, in the absence of extracellular calcium ions, as do various imidazoles and imidazolines.

Cyclic AMP and adenosine

Methylxanthines, and notably theophylline, are inhibitors of the phosphodiesterase which hydrolyses cyclic 3′,5′ adenosine monophosphate (cyclic AMP) to adenosine 5′ monophosphate (Butcher & Sutherland, 1962). An inhibition of the enzyme would result in the accumulation of cyclic AMP in the cell, and has been considered to underlie the potentiation of contraction in the mammalian heart caused by the methylxanthines, by affecting the uptake of calcium by the sarcoplasmic reticulum (Entman, Levey & Epstein, 1969).

On the basis of the present results, cyclic AMP would be expected to induce contractures in frog heart merely because it contains imidazole as a part of its molecular structure. The contracture evoking ability of the more permeable, but equally potent relative of cyclic AMP, N^6 monobutyryl 3′,5′-cyclic AMP has been compared with that of adenosine. The latter is more potent (Table 3). This result supports our suggestion that cyclic AMP does not play a role in mediating the action of the methylxanthines or related compounds in these conditions. An increase in calcium influx associated with cyclic AMP is ruled out, because the contractures persist even when $[Ca]_o$ is reduced below 10^{-8} M.

The antagonistic action of local anaesthetics

The contractures evoked by caffeine in sodium-free fluids can be blocked by appropriate concentrations of procaine and tetracaine (Chapman & Miller, 1974). A concentration of local anaesthetic that inhibits the caffeine response also blocks the response to naphazoline and 1-methyl imidazole. This antagonism suggests a common mode of action for each of these compounds.

Control experiments

The ability of pilocarpine and phentolamine to evoke contractures could mean that a significant release of acetylcholine or adrenaline, from their stores within the heart, is involved in the response to the chemicals used in the present work. Imidazole causes the release of acetylcholine in isolated mammalian pancreas (T. Scratcherd, personal communication). The compounds, acetylcholine 10^{-3} M, atropine 10^{-5} M, propanolol 10^{-5} M and dibenamine 10^{-5} M, failed to induce a tension change and their presence did not affect the response induced by caffeine. These findings rule out the mediation of sympathetic or parasympathetic transmitter chemicals in the contractile events described in this paper.

The influence of anti-histamines

Histamine can initiate contractures in frog heart. Mepyramine, promethazine and dipenhydramine HCl up to 10^{-5} M (well above their pharmacological dose) have no effect on the caffeine contracture, suggesting that

the contracture induction by histamine is not related to its other pharmacological activities. At higher concentrations (10^{-4} M) these antihistamines induce a redevelopment of tension following a sodium-free contracture, without altering a control caffeine response. The antihistamines, which are chemically similar to each other, do not contain the imidazole moiety that has been proposed as the reactive centre of caffeine-like compounds. However, antozoline, an active imidazoline, has antihistamine properties and some common features in the reaction of these other types of compounds cannot be totally excluded. The antihistamines tested resemble chlorpromazine, which is known to disrupt calcium transport in isolated sarcoplasmic reticulum (Balzer, Makinose & Hasselbach, 1968), and to inhibit other transport ATPases, in red blood cells (Mircerova & Simonova, 1971) and brain microsomes (Akera & Brody, 1972). Chlorpromazine will also induce contractures in frog heart (R. A. Chapman, unpublished).

DISCUSSION

It has been shown that a wide variety of cyclic nitrocarbons related to imidazole are able to initiate a redevelopment of tension following the spontaneous relaxation of the contracture elicited by perfusion with high-potassium or sodium-free Ringer. This ability is not related to the release of catecholamines or acetylcholine from the heart tissue, nor to the reduction of the surface tension that is the result of adding methylxanthines to aqueous solutions.

It has been argued that caffeine does not act by increasing the influx of calcium from the bathing medium, because caffeine contractures can be evoked in the effective absence of bathing calcium ions (Chapman & Miller, 1974). This observation has been extended to imidazole and the imidazolines as well as some nucleotides and other active compounds. Large changes in membrane potential are also relatively ineffective in inducing tension development in sodium-free conditions (Chapman, 1973*a*). It would appear, therefore, that the contractures observed in the present work are due to some specific reaction which releases calcium into the sarcoplasm from an intracellular store. This is also suggested by previous work on this tissue (Chapman, 1973*a*, *b*; Chapman & Ellis, 1973; Chapman & Miller, 1974). The most likely structure, for this store, is the sarcoplasmic reticulum because experiments show that calcium is released from isolated mammalian skeletal and cardiac muscle sarcoplasmic reticulum by those methylxanthines, imidazoles and imidazolines that induce contractures in frog heart and not by the 9-methylxanthines and the 2-imidazolidones (R. A. Chapman & N. G. Rutherford, in preparation).

The generality of the present findings is reinforced by experiments on

isolated rat and guinea-pig ventricular trabeculae, which show that contractures can be evoked in normal Ringer by the same range of compounds that are 'active' in the present work (Chapman & Léoty, 1974).

The finding that histidine and imidazole can induce contractures is particularly worrying because these substances are widely used as buffers in experiments with isolated sarcoplasmic reticulum.

The contractures evoked by the methylxanthines and allied compounds in frog heart, would not seem to be directly the consequence of a build up of cyclic AMP within the muscle cells, due to the inhibition of the phosphodiesterase, for the following reasons.

(1) The various methylxanthines have widely differing potencies as phosphodiesterase inhibitors: 1-methyl, 3-isobutylxanthine is some 20–30 times more potent than theophylline, while other methylxanthines are considerably less potent than theophylline (Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970; Beavo, Rogers, Crofford, Baird, Hardman, Sutherland & Newman, 1971), but all have a similar potency in evoking contractures (Fig. 2).

(2) Imadozole, which also elicits contractures, enhances rather than inhibits the activity of the phosphodiesterase (Butcher & Sutherland, 1962; Kukovetz & Pöch, 1967).

(3) The imidazolidones, particularly the benzyl-2-imidazolidones, are very potent phosphodiesterase inhibitors, being some 5000 times more potent than theophylline (Sheppard & Wiggan, 1970) but they do not initiate significant contractures.

(4) Adrenaline, which is widely believed to stimulate cyclic AMP production (Bowman & Nott, 1969), fails to produce contractures.

(5) The responses to adenosine and monobutyryl cyclic AMP are similar.

(6) Cyclic AMP, in the presence of certain protein kinases increases the rate of calcium uptake by isolated cardiac sarcoplasmic reticulum (W. G. Nayler, personal communication).

The release of calcium by cyclic AMP from other structures, such as the cell membrane would also seem unlikely, because the concentration of monobutyryl cyclic AMP that induces a contracture is some 10^5 times the maximum concentration of cyclic AMP found naturally within cells (Robison, Butcher & Sutherland, 1968), and this contracture, that can be elicited in the absence of extracellular calcium ions, is smaller than that evoked by adenosine, and much smaller than that caused by caffeine.

If, as the present work suggests, the imidazole moiety in general, and the double bonded nitrogen atom in particular, confer the contracture promoting ability, then adenosine and cyclic AMP would be expected to produce contractures. A clear-cut answer to this question could perhaps be obtained by testing the effect of the 9-methylxanthines on the activity

of the phosphodiesterase. Beavo *et al.* (1970, 1971) found that a compound of this type, which was extensively substituted elsewhere in the molecule, had little effect on phosphodiesterase activity. Simpler 9-methylxanthines, at 5 mM, do not inhibit this enzyme (N. Amrhein & G. Bitter, personal communication). The same feature of the molecular architecture of the methylxanthines may, therefore, be responsible for the inhibition of the phosphodiesterase enzyme and for the initiation of the contractures. If these two actions are indeed separate, as suggested above, then the similarity of the chemical reactions involved could account for the current dilemma in interpreting the role of cyclic AMP in the initiation of cardiac contraction.

Some conclusions can be drawn about the chemical reactions in which caffeine and the other tension inducing chemicals are involved and how the uncombined 9-position nitrogen (or its equivalent in the imidazole compounds) participates in this reaction. The rapid reversibility of the action of caffeine, probably means that the reaction between the alkaloid and its 'target' site is relatively weak. This must preclude any type of covalent bonding where the two spare electrons in the outer valency orbital of the 9-position nitrogen are shared with the atoms at the target molecule. The evidence supports some sort of electrostatic bonding, like hydrogen bonding, as the most likely type of interaction. This idea would be consistent with the currently accepted organization of the methylxanthine molecule as proposed by Cavalieri, Fox, Stone & Chang (1954). These authors suggest that the uncombined 9-position nitrogen in the xanthine molecule will become a region of negativity in aqueous solutions. Caffeine cannot therefore be directly displacing calcium ions by some sort of ionic substitution. When combined, the 9-position nitrogen will become positively charged with respect to the 7-position nitrogen. The inability of the 7-position nitrogen, when it is the more negatively charged, to become involved in the same chemical reaction as the uncombined nitrogen at the 9-position, may be due to the adjacent double-bonded oxygen group. These oxygens will also polarize in aqueous solutions, the resulting negative polarity could then interfere with any electrostatic bonding of the 1 or 3 as well as the 7-position nitrogens. This hypothesis could be tested by comparing the response of the muscle to a series of methyl phenolimidazoles, but as yet we have been unable to obtain such chemicals.

Any further speculation based upon the results in this paper is unwarranted, as the site of action of caffeine and the other similar chemicals, is more readily studied with isolated sarcoplasmic reticulum where direct measurement of the calcium binding and uptake can be made.

The release of intracellular calcium might be involved in the other pharmacological activities of some of the imidazole-like compounds used

in the present work, such as pilocarpine, histamine, etc. particularly as the liberation of transmitter chemicals from nerve terminals is dependent on the intracellular calcium concentration (Dodge & Rahamimoff, 1967; Katz & Miledi, 1969). In fact recently, caffeine has been shown to affect neuromuscular transmission in the rat in a way consistent with this idea (Wilson, 1973).

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